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CUTTING EDGE

Cutting Edge: L-Selectin (CD62L) Expression Distinguishes Small Resting Memory CD4$^+$ T Cells That Preferentially Respond to Recall Antigen

Richard L. Hengel,[1]† Vishakha Thaker,† Mark V. Pavlick,* Julia A. Metcalf,‡ Glynn Dennis Jr.,§ Jun Yang,§ Richard A. Lempicki,§ Irini Sereti,† and H. Clifford Lane†‡*

Naive CD4$^+$ T cells use L-selectin (CD62L) expression to facilitate immune surveillance. However, the reasons for its expression on a subset of memory CD4$^+$ T cells are unknown. We show that memory CD4$^+$ T cells expressing CD62L were smaller, proliferated well in response to tetanus toxoid, had longer telomeres, and expressed genes and proteins consistent with immune surveillance function. Conversely, memory CD4$^+$ T cells lacking CD62L expression were larger, proliferated poorly in response to tetanus toxoid, had shorter telomeres, and expressed genes and proteins consistent with effector function. These findings suggest that CD62L expression facilitates immune surveillance by programming CD4$^+$ T cell blood and lymph node recirculation, irrespective of naive or memory CD4$^+$ T cell phenotype. The Journal of Immunology,* 2003, 170: 28–32.

Complex organisms compartmentalize cells with like functions, keeping cell numbers to a minimum while creating efficiencies for cell-cell and cell-extracellular matrix interactions. This is the basis for organ systems. Each organ system has its own unique strategy of cell compartmentalization that best suits its function. As a result, cell function can be inferred from patterns of cell compartmentalization. Indeed, immune surveillance, the function of small round lymphocytes, was inferred from experiments first showing that these cells recirculate between blood and lymph node (1, 2).

CD62L expression on naive CD4$^+$ T cells is required for their efficient recirculation and compartmentalization between blood and lymph node (3–6). Through rolling adhesion, CD62L decelerates lymphocytes by engaging ligands expressed on high endothelial venules. A subsequent signaling cascade, beginning with triggering of lymphocyte CCR7, leads to firm arrest and lymphocyte transmigration into the lymph node (7). After scanning dendritic cells naive CD4$^+$ T cells not encountering their cognate Ag return to blood via efferent lymphatics where they continue blood and lymph node recirculation (8, 9). Until recently, memory CD4$^+$ T cells were thought to lack CD62L expression, but this molecule is now known to be expressed on a major subset of memory CD4$^+$ T cells (10–12). Why CD62L is expressed on this subset is unknown, but it may simply be that CD62L serves the same purpose on memory as it does on naive CD4$^+$ T cells: to facilitate recirculation between blood and lymph node for the purposes of immune surveillance. Optimizing dynamic lymphocyte-dendritic cell interactions while limiting cell numbers would seem to be as efficient a mechanism of immune surveillance for memory CD4$^+$ T cells years or decades removed from cognate Ag encounter as for CD4$^+$ T cells naive to cognate Ag encounter. To examine this hypothesis, we examined the functional and genetic characteristics of CD4$^+$ T cells separated on the basis of CD62L expression.

Materials and Methods

Lymphocyte isolation

Human PBMC were obtained by density gradient centrifugation. Magnetic bead positively selected (CD4 mAb) or negatively selected CD45RA, CD8, CD16, CD36, CD56, and CD11a mAbs) CD4$^+$ T cells were then separated into CD62L$^{+/-}$ fractions using anti-PE magnetic beads (Miltenyi Biotec, Auburn, CA). Positively selected CD4$^+$ T cells included CD45RA$^+$ cells while negatively selected CD4$^+$ T cells excluded them. CD4$^+$ T cell separations were >95% pure while CD62L$^{+/-}$ purities were >95% and >85%, respectively.

Lymphocyte proliferation assays (LPA)

Unseparated PBMC (200,000 cells/well) and CD4$^+$ (positively selected) CD62L$^{+/-}$ T cells (150,000 cells/well plus 100,000 irradiated autologous PBMC) were cultured in 96-well plates for 5 days. Tetanus toxoid (TT)[2] 4 μg/ml; Aventis Pasteur, Swiftwater, PA) or CMV (2.5 μl/ml; BioWhittaker, Walkersville, MD) proliferation was measured for 5 TT-immunized CMV-seropositive subjects using 6-h [^{3}H]thymidine (4 μCu/ml) incorporation. Similar responses were measured at days 2, 3, 4, and 5 for three individuals. Duplicate average cpm were measured for triplicate wells and expressed by subtracting average media cpm from average Ag-stimulated cpm (Δcpm).

*Division of Infectious Diseases, Department of Medicine, Georgetown University School of Medicine, Washington, DC 20053; †Clinical and Molecular Retrovirology Section, Laboratory of Immunoregulation, and ‡Office of the Clinical Director, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and §Laboratory of Immunopathogenesis and Bioinformatics, Science Applications International Corporation, Frederick, MD 21702

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[1] Address correspondence and reprint requests to Dr. Richard L. Hengel, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 11B05, Building 10, 9800 Rockville Pike, Bethesda, MD 20892. E-mail address: rhengel@nih.gov

[2] Abbreviations used in this paper: TT, tetanus toxoid; TRF, telomere restriction fragment length; LPA, lymphocyte proliferation assay; FSC, forward light scatter; SSC, side light scatter.

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Telomere restriction fragment (TRF) length analysis

TRF length analysis was performed on $CD4^+$ (positively selected) $CD62L^{+/-}$ T cells or $CD4^+$ (negatively selected) $CD45RA^-CD62L^{+/-}$ T cells, as previously described (13).

cRNA preparation for oligonucleotide arrays

Total RNA was isolated (10–58 μg) from the equal numbers ($8–10 \times 10^6$) of $CD4^+$ (negatively selected) $CD45RA^-CD62L^{+/-}$ subsets from nine donors using RNeasy kit (Qiagen, Valencia, CA). First- and second-strand DNA synthesis reactions were done with the Superscript Choice System (Life Technologies, Frederick, MD) followed by in vitro transcription (Enzo Diagnostics, Farmingdale, NY) using biotin-labeled dNTPs. Complementary RNA samples were fragmented and hybridized to the Affymetrix Human Genome U95A oligonucleotide array. Chip-to-chip normalization and gene hybridization intensity were conducted using Microarray Analysis Suite 4.0 (Affymetrix, Santa Clara, CA). Genes selected were prefiltered: average differences <20 were truncated to 20; mean average difference between comparison groups was set at >30; mean average difference between comparison groups was set at >1.6-fold or <−1.6-fold; Student's *t* test was set at $p < 0.05$. Genes exceeding prefilter thresholds were analyzed with significance analysis of microarray software (14). T scores were generated after 5000 randomizations of the data. A median false discovery rate of <5% was used to identify genes differentially expressed between $CD62L^{+/-}$ memory $CD4^+$ T cells. Readers unfamiliar with National Center for Biotechnology Information official gene symbols are referred to www.ncbi.nlm.nih.gov/locuslink/.

Results and Discussion

To address the hypothesis that CD62L expression by $CD4^+$ T cells facilitates immune surveillance within the afferent limb of the cellular immune response, we measured the function of $CD4^+$ T cells separated into $CD62L^+$ and $CD62L^-$ fractions. We measured the ability of separated cells to proliferate in response to stimulation with TT, as a prototype recall Ag. We compared these results to parallel results after stimulation with CMV Ag, as a prototype recent Ag. We found that proliferation to TT was almost exclusively confined to $CD4^+$ T cells expressing CD62L, while responses to CMV Ags were found in both pools of cells (Fig. 1, *A* and *B*). Subset TT response kinetics were not different on days 2, 3, 4, and 5 (data not shown).

Because recall proliferation to TT was confined to $CD4^+$ T cells expressing CD62L, we next determined whether these cells were also capable of mediating effector functions. To measure effector function, we measured the IFN-γ expression of whole blood-stimulated $CD4^+$ T cells after a brief (6 h) Ag exposure period, as previously described (15). We found that IFN-γ expression after TT stimulation was not different from background. Conversely, we found that IFN-γ expression after CMV stimulation was higher than background (Fig. 1*C*). After successful Ag encounter, some or all responding naive and memory $CD4^+$ T cells down-regulate CD62L, while at the same time they up-regulate other adhesion molecules required for effector cell access into local sites of inflammation (16–19). We further characterized IFN-γ^+ cells and found that they were all confined to the $CD62L^-$ memory $CD4^+$ T cell pool, and that for most cells this did not result from CD62L down-regulation during the Ag stimulation period (Fig. 1, *C* and *D*). Because TT exposure is remotely associated with immunization, while CMV exposure is ongoing, our results suggest that IFN-γ expression is found among cells temporally proximate to

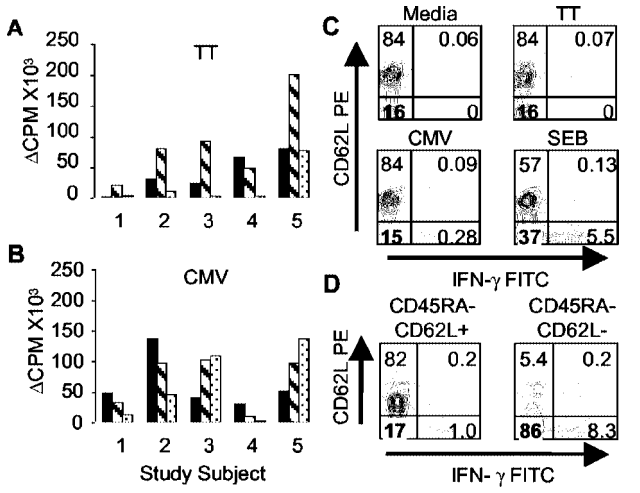

FIGURE 1. Memory $CD4^+$ T cell functional responses vary according to CD62L expression. *A*, TT LPA measured for PBMC (■) was confined to $CD62L^+$ (▨) rather than $CD62L^-$ (▩) $CD4^+$ T cells, for each of five individuals. *B*, Conversely, CMV LPA measured for PBMC (■) was found in both $CD62L^+$ (▨) and $CD62L^-$ (▩) $CD4^+$ T cell subsets. *C*, Whole blood-gated $CD4^+$ T cell IFN-γ responses for one representative individual show that TT responses were not different from background, while CMV responses were 0.03 and 0.28% above background in $CD62L^+$ and $CD62L^-$ fractions. Staphylococcal enterotoxin B responses are shown as positive control. *D*, Previously separated $CD62L^+$ and $CD62L^-$ memory ($CD45RA^-$) $CD4^+$ T cells from a second representative individual were labeled, resuspended in whole blood, and subsequently stimulated with CMV. Gated $CD4^+$ T cells show IFN-γ expression was increased >800% in $CD62L^-$, compared with $CD62L^+$, fractions. Even in the $CD62L^+$ fraction, IFN-γ^+ T cells were $CD62L^-$. Data in *C* and *D* are representative of results from three individuals.

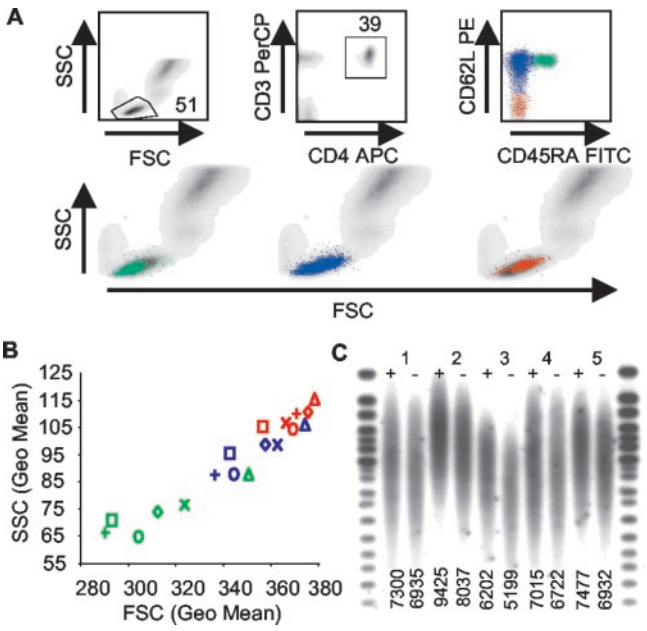

FIGURE 2. $CD4^+$ T cell relative size, complexity, and replicative history vary according to CD45RA and CD62L expression. *A*, Lymphocyte gated PBMC (51%), then $CD3^+CD4^+$ gated T cells (39%), were analyzed for CD45RA and CD62L expression: $CD45RA^+CD62L^+$ (green), $CD45RA^-CD62L^+$ (blue), and $CD45RA^-CD62L^-$ (red). These three subsets were back-gated onto the original lymphocyte gate to show their relative population forward light scatter (FSC) and side light scatter (SSC) (*bottom panel*). *B*, Mean population FSC and SSC values were calculated for each of the three subsets defined in *A* for six individuals. A unique symbol represents each individual and mean FSC and SSC showed that for each; $CD45RA^+CD62L^+$ (green) < $CD45RA^-CD62L^+$ (blue) < $CD45RA^-CD62L^-$ (red). *C*, TRF lengths were compared for five individuals (labeled 1–5). In each case, average TRF lengths (numerical average below each lane) were longer in the $CD62L^+$ (labeled +) compared with $CD62L^-$ (labeled −) $CD4^+$ T cell pools, regardless of whether naive ($CD45RA^+$) cells were present in the fraction (individuals 1–3) or not (individuals 4–5).

cognate Ag encounter in vivo. Further, because TT induces IFN-γ gene and protein expression with longer Ag exposures in vitro, this suggests that these TT-specific IFN-γ$^+$ cells arise ex vivo from CD62L$^+$ memory CD4$^+$ T cell precursors (20).

In addition to down-regulating CD62L, CD4$^+$ T cells that successfully recognize their cognate Ag become activated, enlarge, develop greater cytoplasmic complexity, and begin to divide (17, 18). If CD62L$^+$ memory CD4$^+$ T cells are precursors for CD62L$^-$ memory CD4$^+$ T cells in vivo, then this latter subset should be larger, have greater cytoplasmic complexity, and have evidence of more rounds of cell division. To determine whether this was true, we first compared the relative size and complexity of CD4$^+$ T cells distinguished on the basis of their CD62L expression. To measure relative cell size and complexity, we measured the reflective and refractive properties of memory and naive CD4$^+$ T cell subsets using flow cytometry (21). We found that that CD62L$^-$ memory CD4$^+$ T cells were largest and most complex, whereas naive CD4$^+$ T cells were the smallest and least complex and CD62L$^+$ memory CD4$^+$ T cells were intermediate between the two (Fig. 2, *A* and *B*).

To examine replicative history, we measured the relative average TRF lengths of these subsets. Telomeres are located at the distal ends of chromosomes, functioning to preserve chromosomal integrity during mitosis. They shorten with successive rounds of cell division (22). We found that memory CD4$^+$ T cells expressing CD62L had longer telomeres than CD4$^+$ T cells not expressing CD62L (Fig. 2*C*). No difference in telomerase mRNA expression was found between CD62L$^+$ and CD62L$^-$ subsets using microarray analysis, although low-level expression (44–712 mRNA copies/10^6 cells) was found in both pools for 5 of 10 individuals using real-time quantitative PCR (data not shown). Less than one gene copy per thousand cells is unlikely to account for the TRF length differences we found (Fig. 2*C*).

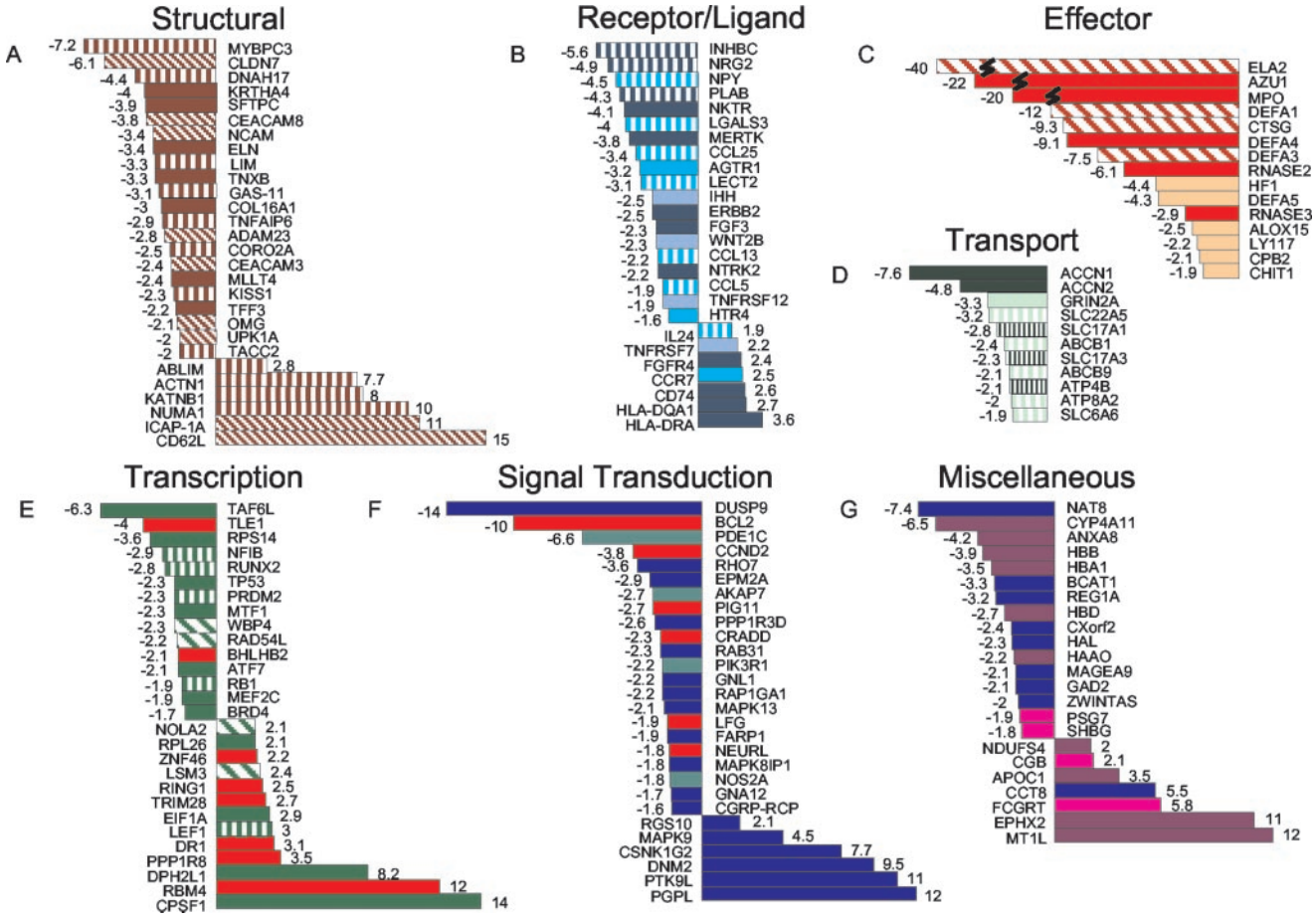

FIGURE 3. Genes differentially expressed between CD62L$^+$ and CD62L$^-$ memory CD4$^+$ T cells. Fold increases are shown for CD62L$^+$ (bars to the right, positive numbers) and CD62L$^-$ fractions (bars to the left, negative numbers). Labels reflect National Center for Biotechnology Information gene symbols. *A*, Structural genes were subdivided into three groups: extracellular matrix (solid red bars), cytoskeletal (vertical lines) and membrane gene products (diagonal lines). *B*, Receptor and receptor ligand genes were subdivided into chemokine and cytokine (turquoise), cell fate and apoptosis (light blue), or trophic and growth factor gene product pathways (dark blue). Solid bars indicate receptors while vertical lines indicate ligands. C, Effector genes were subdivided into myeloid-type (red) and other gene products (tan). Solid red bars indicate genes that have yet to be reported, and diagonal lines indicate genes that have already been reported, among lymphocyte subsets. *D*, Transport genes were subdivided into amino acid and xenobiotic (light green) or cation transporting gene products (dark green). Vertical lined bars indicate active transporters while solid bars indicate passive transporters. *E*, Transcription genes are subdivided into transcriptional or translational repression (red), promotion (green), or both function gene products (green diagonal). Vertical lines indicate RNA splicing and DNA repair genes. *F*, Signal transduction genes are subdivided into cell cycle and apoptosis (red), MAPK (blue), or second messenger gene product pathways (green). *G*, Miscellaneous genes were subdivided into oxidative stress and lipid peroxidation (purple), sex hormone-related (pink), and other gene products (blue).

Because recall and effector responses by memory $CD4^+$ T cells correlated with the presence or absence of CD62L expression, and because cell size, complexity, and replicative history of these cells were all consistent with $CD62L^-$ cells arising from $CD62L^+$ $CD4^+$ T cells, we next examined whether differential gene expression patterns supported these functional and phenotypic differences (14). On average, we found twice as much RNA per million cells in $CD62L^-$ compared with $CD62L^+$ fractions, consistent with a more transcriptionally active $CD62L^-$ fraction. To measure differential gene expression, we used oligonucleotide microarrays to compare $CD62L^+$ to $CD62L^-$ memory $CD4^+$ T cells.

Not surprisingly, CD62L transcripts were highly expressed in the $CD62L^+$ fraction, as were CCR7 and CD27 (TNFRSF7), both frequently coexpressed with CD62L (Fig. 3, *A* and *B*) (23). This fraction had no genes represented in the effector and transport categories (Fig. 3, *C* and *D*). The greatest number of genes in the $CD62L^+$ fraction was found among the category of genes modulating transcription and translation. Most of these function to repress RNA or DNA transcription (Fig. 3*E*), consistent with relative transcriptional quiescence in this pool. Several signal transduction genes were increased, including genes in the protein kinase C (PTK9L and CSNK1G2) and c-jun N-terminal kinase (MAPK9) pathways (Fig. 3*F*). Of interest was increased expression of metal binding and oxidative stress genes (Fig. 3*G*). Together, these transcripts and their proteins identify potentially important regulatory mechanisms and therapeutic targets that may be relevant to manipulating $CD62L^+$ memory $CD4^+$ T cells for therapeutic ends.

Within the $CD62L^-$ fraction there was exclusive expression of genes encoding effector and small molecule transport proteins (Fig. 3, *C* and *D*). Several defensins, elastase, and cathepsin G gene products have already been described in lymphocyte subsets, suggesting that these and other myeloid-type gene transcripts have distribution beyond myeloid cells (24–26). Expression of genes that encode extracellular matrix, cytoskeletal, and cell adhesion proteins, which all facilitate cell-cell, cell-extracellular matrix and locomotory functions, was also an important finding and consistent with effector functions at local sites of inflammation (Fig. 3*B*). Indeed, we found monocyte-attracting (LGALS3 and CCL13 and lymphocyte-attracting (CCL5 (RANTES), CCL25, LECT2 and NPY) chemokine genes, also consistent with this idea. Similarly, we found growth inhibitory genes (TGF-β superfamily members PLAB and INHBC), stimulatory growth factor (NRG2 and FGF3) and growth factor receptor genes (ERBB2 and NTRK2), implicated in autocrine and paracrine trophic mechanisms, all consistent with a pool of cells capable of engaging in effector functions (Fig. 3*B*). The presence of cell-fate (WNT2B, TLE1, and IHH), regulatory (MEF2C, RUNX2, and TAF6L), cell-cycle and apoptosis pathway genes (RB1, TP53 (p53), BCL2, and CRADD) further implicated the $CD62L^-$ pool of cells as a more recently activated, differentiated, and expanded subset of cells downstream of immune surveillance and TCR signaling (Fig. 3, *B*, *E*, and *F*). As with genes identified in the $CD62L^+$ subset, these transcripts and their proteins identify potentially important regulatory mechanisms and therapeutic targets that may be relevant to manipulating $CD62L^-$ memory $CD4^+$ T cells for therapeutic ends.

In summary, we have shown that the expression of CD62L distinguishes memory $CD4^+$ T cells that proliferate in response to stimulation with recall Ag. Conversely, its absence distinguishes memory $CD4^+$ T cells capable of rapidly producing cytokine after stimulation with a more recently encountered cognate Ag. We have also shown that $CD62L^-$ cells likely arise from $CD62L^+$ memory $CD4^+$ T cells (not precluding their generation from naive $CD4^+$ T cells) and that these two subsets have novel and important differences in their patterns of gene expression, consistent with their functional characteristics. These results were predicted from studies implicating the preferential depletion of $CD4^+CD62L^+$ T cells as a general mechanism of HIV-1 pathogenesis (27, 28). They are also similar in some ways to those from a study using CCR7 expression to distinguish staphylococcal enterotoxin B-stimulated $CD4^+$ T cells producing IFN-γ (12). However, these authors did not find that CCR7 expression distinguishes $CD4^+$ T cells responding to TT. This, along with recent reports finding effector function in $CCR7^+$ fractions of T cells, makes it unclear whether CCR7 expression unambiguously distinguishes between memory and effector $CD4^+$ T cells (23, 29, 30).

Distinguishing immune surveillance from effector $CD4^+$ T cells is critical to distinguishing qualitative from quantitative mechanisms that affect immune assays that measure global $CD4^+$ T cell function. Accounting for these cells will be important to identifying new surrogate markers relevant not only to measuring the efficacy of drugs and vaccines that manipulate $CD4^+$ T cell immune function for therapeutic ends, but also to help in their design by suggesting new treatment strategies and new therapeutic targets. Our results suggest that CD62L expression can be used to distinguish immune surveillance from effector functions and that this reflects the role that CD62L has in compartmentalizing these cells in vivo, an example of how form reflects function in organic systems.

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